S-nitrosoglutathione influences the activity of μ- and m-calpain

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Introduction

Nitric oxide is a free radical that can be released in many tissues in vivo including muscle. S-nitrosylation is the covalent attachment of a nitrogen monoxide group to thiol side chain of cysteine (Hess et al., 2005). Nitric oxide induced S-nitrosylation has been considered as one of the important mechanisms for post-translational regulation of protein functions (Jaffrey et al., 2001). The formation of S-nitrosothiols and mixed disulfides has been described to have inhibitory effects on the catalytic activity of cysteine proteases in vivo and vitro (Ascenzi et al., 2001).

It is well known that calpain system is responsible for the degradation of cytoskeletal and intermediate proteins during postmortem storage resulting in tenderization (Koohmaraie, 1992; Huff-Lonergan et al., 1996). The calpain system is composed of multiple protease isoforms including two calcium-dependent cysteine proteases μ- and m-calpain and their inhibitor calpastatin (Goll et al., 2003). Oxidation can inhibit their proteolytic activity (Carlin, et al., 2006). Nitric oxide has been reported to decrease the proteolytic activity of m-calpain and μ-calpain. This inhibition can be reversed by dithiothreitol (Michetti et al., 1995).

In the present study, we hypothesized that nitric oxide affects the activity of μ- and m-calpain. This may be due to S-nitrosylation of cysteine residues. S-nitrosoglutathione (GSNO) was chosen as nitric oxide donor in this study because it is a natural compound and plays critical roles in nitrosylation in vivo.

Materials and Methods

μ-Calpain and m-calpain. Calpains were purified from skeletal muscle according to the methods of Thompson and Goll (2000) with minor modifications. For the study, calpain was incubated in 50 mM HEPES adjusted by NaOH to the appropriate pH. The concentration of S-nitrosoglutathione (GSNO, Sigma, US) was added at a ratio of 2:1 (w:w) GSNO to calpain in corresponding treatments. The concentrations of calcium were either 0 or 1 mM. The treatments for μ-calpain were (in order of addition): control: μ-calpain; GSNO: μ-calpain +GSNO; $CaCl_2$: μ-calpain+$CaCl_2$; $CaCl_2$+GSNO: μ-calpain+$CaCl_2$+GSNO; GSNO+ $CaCl_2$: μ-calpain+GSNO +$CaCl_2$. The amount of μ-calpain was 28.7 μg in each treatment. The pH used was 7.5 for μ-calpain.

The five treatments for m-calpain were (in order of addition): control: m-calpain; GSNO: m-calpain+GSNO; $CaCl_2$: m-calpain+$CaCl_2$; $CaCl_2$+GSNO: m-calpain+$CaCl_2$+GSNO; GSNO+$CaCl_2$: m-calpain+GSNO+$CaCl_2$. The amount of m-calpain was 32.76 μg in each treatment. The pH used was 6.5 for m-calpain. The reactions were done for 60 minutes on ice. The protein concentration was measured after incubation for each treatment using Molecular Devices (THERMO max, US) with Bio-Rad protein assay (Bio-Rad, US).

Calpain activity assay. The activity of calpain was measured as described by Koohmaraie (1990). To study whether the nitrosylation of calpain could be reversed by mercaptoethanol (MCE), the casein buffer for the assay was made with MCE or without MCE. The final activities of μ-calpain and m-calpain were calculated as specific activity (U/mg). One unit (U) of activity is defined as one increase of one absorbance unit at 278 nm after 60 min incubation at 25 ^{0}C.

Data analysis. All data were analyzed using SAS version 9.1 (NC, US) and significance was reported at the $P<0.05$ level. General Linear Procedure and analysis of variance (ANOVA) were used to determine the significance of the effect of GSNO and calcium.

Results and Discussion

m-Calpain activity. There was no significant difference for m-calpain activity between treatment with the addition of GSNO alone and control group in either casein buffer (with or without MCE). This showed that the GSNO may not have significant effects on the activity of m-calpain in the absence of calcium at pH=6.5. After the addition of calcium chloride, the activity of m-calpain was not significantly different with control and GSNO group ($P>0.05$). However, the m-calpain activity was significantly decreased by GSNO in the presence of calcium in both the assays with MCE and without MCE ($P<0.05$). The addition of GSNO in the solutions significantly decreased the m-calpain activity regardless of whether GSNO or calcium were added first to the calpain ($P<0.05$). These results were in agreement with the previous report by Michetti et al. (1995) who showed S-nitrosylation by sodium nitroprusside can inactivate of m-calpain when the pH was shifted to acid conditions.

μ-Calpain activity. Similar to the m-calpain activity, GSNO did not significantly influence the activity of μ-calpain in the absence of calcium at pH=7.5. With MCE in casein activity assay, the treatment with the addition of calcium alone had lower activity than two treatments without calcium ($P<0.05$). This may be due to the rapid

autolysis of µ-calpain in the presence of calcium (Suzuki et al., 1981). Interestingly, the activity of µ-calpain in the treatment with the addition of GSNO first and then addition of calcium was significantly higher than the treatment with just addition of calcium in MCE group ($P<0.05$). The observation that some activity is restored with inclusion of MCE in the assay suggests that inhibition by GSNO is reversible. Without MCE in casein buffers, treatments with the addition of calcium and calcium and GSNO had lower µ-calpain activity than treatments without calcium ($P<0.05$). The treatment with addition of GSNO first and then adding calcium had higher activity than treatment with just addition of calcium and the treatment of addition of calcium and GSNO in that order ($P<0.05$). The higher activity of GSNO+$CaCl_2$ group may be due to the slower rate of autolytic inactivation caused by GSNO (data not shown).

Table 1. Effects of GSNO on the activity of m-calpain under different calcium concentrations at pH=6.5

Calpain	With or without MCE in casein buffer	Treatment*				
		Control	GSNO**	$CaCl_2$***	$CaCl_2$+GSNO	GSNO+$CaCl_2$
m-calpain	MCE	79.83[a]	82.33 [a]	79.50[a]	52.17[b]	48.33[b]
pH=6.5	Without MCE	54.33[a]	54.83[a]	54.83[a]	33.67[b]	35.17[b]

[a–b] Means in the same row without a common superscript letter differ significantly ($P < 0.05$).

*Treatment (in order of addition): control: m-calpain; GSNO: m-calpain+GSNO; $CaCl_2$: m-calpain+$CaCl_2$; $CaCl_2$+GSNO: m-calpain+$CaCl_2$+GSNO; GSNO+$CaCl_2$: m-calpain+GSNO+$CaCl_2$.

**Concentrations: GSNO: 1.05 mM; ***Concentration: $CaCl_2$: 1 mM;

Table 2. Effects of GSNO on the activity of µ -calpain under different calcium concentrations at pH=7.5

Calpain	With or without MCE in casein buffer	Treatment*				
		Control	GSNO**	$CaCl_2$***	$CaCl_2$+GSNO	GSNO+$CaCl_2$
µ-calpain	MCE	32.84[a]	32.50[a]	22.33[b]	26.57[ab]	30.23[a]
pH=7.5	Without MCE	22.67[a]	23.78[a]	18.22[b]	16.59[b]	22.67[a]

[a–b] Means in the same row without a common superscript letter differ significantly ($P < 0.05$).

*Treatment (in order of addition): control: µ-calpain; GSNO: µ-calpain+GSNO; $CaCl_2$: µ-calpain+$CaCl_2$; $CaCl_2$+GSNO: µ-calpain+$CaCl_2$+GSNO; GSNO+ $CaCl_2$: µ-calpain+GSNO+$CaCl_2$.

**Concentrations: GSNO: 1.19 mM; ***Concentration: $CaCl_2$: 1 mM;

Conclusions

GSNO has significant effects on the activity of µ- and m-calpain in the presence of calcium. For m-calpain, GSNO can further decrease its activity at the pH 6.5 in the presence of calcium. Neither µ- or m-calpain are influenced by GSNO in the absence of calcium. Nitrosylation of calpain proteinases may be an important regulator of calpain mediated processes.

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